4/p/ots
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Title: Potatoes with increased protein content

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The invention relates to the breeding and selection of potatoes.

Apart from being an important staple food, potato is classically the raw material for
industrial production of starch from potato tubers. Furthermore, these days, the
10   industrial harvest of potato protein from potato tubers, earlier seen as a *quantité
négligeable* , attracts more attention considering the increased value given to
vegetable protein sources, for example for animal food, if only as a side product
accompanying starch production. Where, chemically, potato starch in potato tubers
essentially consists of two components: amylopectin and amylose in a proportion of
15   approximately 80% to 20%, potato proteins in tubers essentially consist of protease
inhibitors that help protect the tuber against disease such as parasite infestations
or fungal or bacterial rot and storage proteins such as patatin in a proportion of
approximately 60% to 40%.
For various reasons, starch producers prefer potatoes with different ratios of
20   amylopectin and amylose. An earlier induced gene mutation in potatoes that affects
the synthesis of the enzyme granule bound starch synthase (GBSS), and the
subsequent molecular cloning of this gene (Hovenkamp-Hermelink et al., 1987,
Theor. Appl. Genet. 75:217-221; Visser et al., 1989, Plant Science 64:185-192) has
opened possibilities for altering the starch composition of potatoes – either through
25   established breeding methods or through modern techniques of genetic
manipulation.
The GBSS mutation in potato is similar to the so-called waxy (*wx*) mutation in
maize and prevents the production of amylose, when expression or specific function
of the GBSS protein is absent. Therefore, this mutation has been designated as
30   amylose-free (*amf*) mutant of potato. Herein, the *amf*-gene mutation stands for a
modification of the GBSS-gene that leads to a complete functional loss of GBSS-
activity, notwithstanding that GBSS-like gene products, without the specific
activity, may still be expressed from the gene's transcripts in question, whereby the
Amf-gene stands for a gene from which gene products with GBSS-activity can still
35   be obtained. The *amf*-gene character is determined by a monogenic mendelian

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recessive gene, the phenotype of which can be detected in various plant parts such as columella cells of root tips, tubers, plastids in the stomatal guard cells and in microspores (Jacobsen et al., 1989, Euphytica 44:43-48). When these parts are stained with a potassium iodine solution (Lugol), starch is stained red in mutants
5   and dark blue in the wild type.

Unlike many other phenotypic genetic markers, the mutated or functionally deleted GBBS- or amf-gene offers certain special advantages for genetic analysis as well as for breeding. For example, the progeny can be classified at a very early seedling stage as well as in adult plants, through pollen staining, homo- and heterozygotes
10   can be unambiguously classified: the dosages 2-4 of the mutant allele in a tetraploid can be easily detected through the ratios 5:1, 1:1 and 0:1 in stained pollen samples; different types of 2n-gametes in diploid clones can be detected and their influence on the phenotype and genotype of tetraploid from $4x*2x$ crosses can be predicted.

15   Prospects of using the material in conventional as well as in analytic breeding of potato have since the development of the amf-gene potato mutant of Hovenkamp-Hermelink been opened. A disadvantage for breeding is the recessive nature of amf, which complicates the combination of this character with other agronomic traits at the tetraploid level.

20   Therefore, the analytic breeding method advocated by Chase (1963, J. Genet. Cytol. 5:359-364), which involves breeding of potato at the diploid level and returning to the tetraploid condition through the use of 2n-gametes, could be of considerable value for breeding amf-varieties. The aim of such investigations are at least two fold: a. to combine amfamf and Amfamf genotypes with that of 2n-gamete
25   formation, and b. to create fertile, nulliplex clones as basic material for breeding amylose-free potatoes. On the other hand, development of suitable diploid material that produces high frequencies of 2n-pollen and 2n-eggs would also open the way for unilateral and bilateral sexual polyploidization (Mendiburu and Peloquin, 1976, Theor. Appl. Genet.48:137-143). Such diploid breeding material may be
30   homozygous (amfamf) or heterozygous (Amfamf), because in both cases selection can be carried out based on pollen phenotype.

The invention relates to the breeding and selection of potatoes. Surprisingly, it was found herein that potatoes with at least one amf-allele background have a distinct
35   phenotypic advantage when compared with potatoes having a similar genetic

background lacking the *amf*-gene. One such advantage relates to protein content. Genotypes that are nulliplex for the *Amf*-allele, i.e. for diploid plants the *amfamf* (aa) and for tetraploid plants the *amfamfamfamf* (aaaa) genotypes display said advantage even stronger.

5   The invention provides a potato plant or part derived thereof (such as a cell, a protoplast, a tuber, an embryo, a seed or an explant) having at least one *amf*-gene said potato plant or part further provided with an increased capacity to store a protein (herein also identified as a high protein potato) as characterized by a total raw protein content of its tubers (preferably as determined in the potato juice

10   derived thereof) at least 1.9% m/m, more preferred at least 2.3% m/m, most preferred at least 2.7% m/m. Within a potato homozygous for the *amf*-allele as provided herein, i.e. an amylose-free high protein potato, such increased capacity to store protein is most fully developed. The inventors have gathered the surprising insight that depriving a potato of GBSS-activity allows for increasing protein

15   storage in said potato, provided it has the genetic capacity to produce increased, or at least sufficient, amounts of said protein. Potatoes comprising an *amf*-allele have essentially higher protein storage capacity than potatoes of otherwise similar genetic background having no *amf*-gene. Potatoes homozygous for the *amf*-allele are, speaking from the viewpoint of protein storage, preferred.

20   Originally, the *amf*-mutation was induced in a monohaploid which had been selected only for flowering (Hovenkamp-Hermelink et al., 1987, *ibid*) but not for fertility and agronomic characters. Therefore, in order to incorporate this recessive mutant in other potatoes the inventors crossed the diploid genotype derived from the monoploid mutant clone with agronomically more desirable clones which,

25   however, have the wild type of the *Amf* gene. As a first step in this process, fertile diploids that are homozygous for the mutant character (*amfamf*), were produced. When these diploids are somatically doubled through in vitro adventitious shoot regeneration, the resulting tetraploids proved to be less fertile (both male and female). However the 4x plants obtained through meiotic doubling – using 4x x 2x

30   crosses- gave rise to fertile nulliplex tetraploids. Thus, in spite of high levels of sterility and expression of lethal factors in the initial stages, more fertile and vigorous diploid and tetraploid breeding material were created with the desired *amf*-genotypes. Availability of vigorous, fertile and agronomically useful tetraploid genotypes than led to conventional breeding of *amf*-mutants of potato. It was than

35   surprisingly found that amf-mutants, resulting from crosses with wild-type

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potatoes had increased storage capacity for proteins in their tubers, these days considered an economically desirable trait.

It is preferred that said high total raw protein content is also reflected in the amount of protein that can be harvested, e.g. from the tubers. Such measure is
5   given by identifying the fraction of coagulating protein available for harvest, as further explained in the detailed description. The invention also provides a potato plant or part derived thereof (such as a cell, a protoplast, a tuber, an embryo, a seed or an explant) having at least one *amf*-gene said potato plant or part further provided with an increased capacity to produce harvestable protein as
10 characterized by a total coagulating protein content of its tubers (preferably as determined in the potato juice derived thereof) at least 0.9%, more preferred at least 1.2%, most preferred at least 1.5%. Considering that high protein levels are these days often more profitable than high starch levels, the invention also provides a high protein potato (i.e. with more than 1.2%, preferably more than 1.5%
15 coagulating protein in its tubers) characterized in that its tubers essentially show a coagulating protein versus starch ratio of at least 45 kg/ton, more preferred of at least 90 kg/ton.

Furthermore, the invention provides a high-protein potato according to the invention characterized in that it is a transgenic potato, for example provided with
20 a gene or gene encoding for a heterologous protein, for example with the purpose to provide a high protein potato according to the invention additionally provided with increased levels of essential amino acids. It is preferred that such a heterologous protein comprises a heterologous protein rich in essential amino acids. About half of the 20 amino acids found in proteins can be made by vertebrates; the others
25 must be supplied in the diet. For this reason, the latter are called essential amino acids. These include the strictly essential amino acids which are lysine, leucine, isoleucine, valine, phenylalanine, methionine, threonine and tryptophan. Additionally, tyrosine and cysteine, although they are not strictly essential, must be considered as such, since they are synthesised only from essential amino acids:
30 tyrosine from phenylalanine and cysteine from methionine. In particular, humans and other monogastric animals cannot synthesise the essential amino acids and need to obtain these from their diet. The diet of humans and livestock is largely based on plant material. However, several of these essential amino acids are often only present in low concentrations in crop plants, which mainly constitute said
35 plant based diets. In particular, lysine, threonine, methionine or tryptophane often

lack in such diets. Dietary proteins are often not nutritionally equivalent, which correlates with the amino acid composition of the different proteins. Feeding a diet that provides an inadequate amount of one of the essential amino acids leads to negative nitrogen balance, since the normal catabolism of proteins continues, but

5   new synthesis for replacement is limited by the relative lack of the essential amino acid. This occurs even when the total dietary intake of protein is apparently adequate. The extent to which a dietary protein can be used for the synthesis of tissue proteins is limited by the content of the essential amino acid that is present in an amount relative to the requirement. This is the limiting amino acid of that

10   protein. Now that a high protein potato is provided it is beneficial to use this for the expression and storage of valuable proteins. The invention furthermore provides a transgenic potato cell with at least one *amf*-allele having been provided with a nucleic acid encoding a proteinaceous substance, a sink protein. In a preferred embodiment, said cell accumulates said sink protein up to more than 2%,

15   preferably 4%, or even more than 5% to more than 7% of the total protein content of said cell. The protein preferably contains a high content of essential amino acids (preferably methionine, cysteine, lysine, threonine, or tryptophane). In a preferred embodiment, the invention provides a high protein potato according to the invention comprising a heterologous protein rich in essential amino acids such as

20   listed in Table 4.

The invention also provides a method for breeding and selecting a potato with an increased capacity to store a protein comprising crossing a first parent potato with at least one *amf*-gene with a second parent potato without an *amf*-allele, and selecting progeny for the presence of at least one *amf*-allele and for a protein

25   content of its tubers higher than detected in said first parent or said second parent. It is preferred that progeny is selected for a protein content of its tubers higher than detected in said first parent and said second parent. Of course, the storage of proteins being most fully enhanced in amylose-free plants, it is most preferred to select progeny homozygous for the *amf*-allele.

30   In a preferred embodiment, the invention provides a method for breeding and selecting a potato with an increased capacity to store a protein comprising crossing a first parent potato with at least one *amf*-allele with a second parent potato without an *amf*-gene, and selecting progeny (preferably homozygous for the *amf*-allele) by testing it for the presence of at least one *amf*-gene and testing it for total

35   raw protein content with a method, such as the Kjeldahl method as described

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herein, to determine total raw protein content of its tubers and selecting progeny

with a total raw protein content higher than detected in said first parent or said

second parent, said method preferably further comprising selecting progeny with a

total raw protein content of its tubers higher than detected in said first parent and

5   said second parent.

In a further preferred embodiment, the invention provides a method for breeding

and selecting a potato with an increased capacity to produce harvestable protein

comprising crossing a first parent potato with at least one *amf*-allele with a second

parent potato without an *amf*-allele, and selecting progeny (preferably homozygous

10 for the *amf*-allele) by testing it for the presence of at least one *amf*-allele and

testing it for coagulating protein content with a method comprising determining

total raw protein and total soluble raw protein remaining in solution after a protein

coagulation procedures, such as immersion in a boiling water bath as described

herein, to determine or calculate total coagulating protein content of its tubers and

15 selecting progeny with a total coagulating protein content higher than detected in

said first parent or said second parent, said method preferably further comprising

selecting progeny with a total coagulating protein content of its tubers higher than

detected in said first parent and said second parent.

Furthermore, the invention provides a potato selected with a method according to

20 the invention, use of a potato as provided herein for the industrial production of

starch and/or protein and use of a potato as provided herein in breeding and

selection programmes of potatoes. In particular, the invention provides use of a

potato plant or part derived thereof having at least one *amf*-allele in a breeding and

selection programme directed at providing potatoes with an increased protein

25 content.

Figures 1 to 4.

Examples of breeding schemes for breeding and selecting potatoes

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Detailed description

Using an amylose-free (amf)mutant of diploid potato (*Solanum tuberosum*), diploid

35 and tetraploid clones with different genotypes at the *amf*-locus were produced. In

order to make use of the diploid material in analytic breeding of *amf*-potatoes,
clones were selected that produced considerable frequencies of 2n-pollen and 2n-
eggs. Successful attempts were made to select normal synaptic as well as
desynaptic clones producing 2n-gametes. When for example microspores are

5   stained with a potassium iodide solution (Lugol), starch is stained red in mutants
(comprising only the *amf*-gene) and dark blue in the wild type (comprising only the
A*mf*-allele). Based on the phenotype of starch in the microspores, tetraploid clones
with nulliplex, simplex, duplex, triplex and quadriplex genotypes at the *Amf*-locus
were selected. We investigated starch properties and protein content in various

10   parts of the mutant potato plant. Starch composition and protein content in tubers
turned out to be an easily scorable feature. It allows in breeding programmes for
amylose-free potatoes an early assessment of starch and/or protein composition in
the prospective plants or parts thereof.

15   *Plant material*. Monoploid amylose-free (amf) clone 86.040 and the parent clone
AM79.7322 are described in Hovenkamp-Hermelink et al. (1987, *ibid*). Doubled
amf-plants were obtained by adventitious shoot regeneration on leaf explants,
which were taken from *in vitro* propagated shoots of monoploid 86.040. After root
induction in MS$_{30}$ (Murashige & Skoog, 1962, Physiol. Plant 15:473-497) (MS)

20   medium supplemented with 30 g/l sucrose) a number of these diploid amf-plants
were transferred into a glasshouse, at 19°C at day: 17°C at night and 16 h
daylength, in sterilized leaf containing soil. For better flowering, part of the
doubled plants was grafted onto tomato rootstock. Pollen fertility was estimated,
after aceto carmine staining. For the crosses, a variety of wild-type potato pollen

25   was used. The crosses were made on open flowers of diploid (2x) clones of
86.040The wild-type potato clones had been selected for good male an female
fertility and 2n-gametes (unreduced gamete) production in male and female
parents.
This resulted in breeding material with better fertility both on male and female

30   side giving opportunity to make crosses with more advanced diploid breeding
material. From these crosses diploid as well as tetraploid progeny (4x) was
obtained. Diploid material was segregating for the amf-allele, resulting in 25%
homozygous plants, which could be selected by colouring the tubers with a iodine
solution. Also some tetraploid progeny could be obtained as a result of unreduced

35   pollen and unreduced eggcells in both parents. This bilateral sexual

polyploidization has also been used in a third cycle of crosses, making use of diploid homozygous amf-clones. A second method to achieve tetraploid progeny has been 4x.2x crosses, where only the pollendonor has to form unreduced gametes to get a tetraploid progeny (unilateral sexual polyploidization).

5   In general the first two or three cycles of this breeding program have been used to produce male and female fertile amf-breeding material, on the diploid level with the ability to produce unreduced gametes, as a start for a breeding program on diploid and tetraploid chromosomal level. In these breeding cycles also properties as tuber shape, number of tubers and starch content where observed, but no

10   stringent selection where carried out.
     From the third cycle onwards crosses have been made between homozygous tetraploid amf-clones with existing tetraploid starch potato varieties. From these varieties genetic variation with respect to total starch production and resistance against diseases (potato cyst nematode, late blight, wart disease) was introduced.

15   In the second cycle of these crosses made for agronomic improvement segregation of homozygous recessive amf-clones was expected and colouring of tubers with iodine solution was carried out. As a result of this breeding program some agronomical acceptable clones were produced, which are both useful for the large scale production of amylosefree potato starch and as crossing parents in 4x.4x and 4x.2x

20   crosses.

     Starch analysis. Starch granules in micropores and tubers were stained with I_2-KI solution according to Hovenkamp-Hermelink et al. (1987), in stomatal guard cells and other leaf cells according to the treatment described for, microspores and in

25   root cap cells by treatment of root tips with a mixture of Lugols-solution and choralhydrate (1:1, v/v). Four gram of choralhydrate is dissolved in 2 ml of water. The amylose percentage in starch solutions of tubers was measured according to Hovenkamp-Hermelink et al., 1988, Potato Res. 31:241-246). Roottips were fixed and stained according to Pijnacker and Ferwerda (1985, Can. J. Genet. Cytol.

30   26:415-419) for chromosome counts and karyotypic investigations. When for example microspores are stained with a potassium iodide solution (Lugol), starch is stained red in mutants (comprising only the *amf*-gene) and dark blue in the wild type (comprising only the A*mf*-gene) (Jacobsen et al., 1989, Euphytica 44:43-48).

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Protein analysis

For determining raw and coagulated protein content, 300 grams of tuber material together with 1000 ppm sodium bisulphite was grinded in a laboratory blender, type Waring Blendor. To determine the dry matter content an homogeneous sample
5 of approx. 10 gram was taken and dried overnight at 40°C. The rest of sample was centrifuged for 10 min at 4600 rpm. Of the supernatant raw protein content was determined by determining nitrogen content with the Kjeldahl method and dry matter by overnight drying at 40°C. To determine the coagulated protein content in the supernatant the pH was adjusted to 5.2 with 19% HCl and the liquid was boiled
10 for 1 minute. Subsequently, the samples were centrifuged for 10 min at 10000 rpm. To remove the light substance the above liquid was filtered over an S&S 595 paper filter. Nitrogen content of the supernatant after the coagulation step was determined by the Kjeldahl method. All experiments were carried out in duplicate.

15

Raw and coagulated protein content was calculated as follows:

$$\text{Contribution of juice} = \frac{100 - \%DrySubs\tan cePulp}{100 - \%DrySubs\tan ceJuice}$$

Raw protein = N total x contribution juice x 1.5 x 0.88 x 6.25

20 Coagulated protein = (N total – N after coagulation) x 6.25 x contribution juice x 1.5 x 0.88

(1.5 : dilution factor)

(0.88 : correction factor)

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Embryo culture. Unripe berries were surface sterilized by treatment for 1 minute with 70% alcohol and for 15 minutes with a saturated solution of Ca-hypochlorite, containing a few drops of 1%SDS (sodium dodecylsulphate) solution per 100 ml. The sterilized berries were cut open aseptically. Ovules were collected and cultured
30 on medium EC2 (MS-medium supplemented with 1.10^{-5} g/l kinetin, 1.10^{-6} g/l LAA, 8g/l agar and 30 g/l sucrose) as defined by Neal & Topoleski (1983, J. Amer. Soc. Hort. Sci. 108:434-438; 1985 J. Amer. Soc. Hort. Sci. 110:869-873) for embryo culture of tomato. During ovule culture, the integument rapidly attained a brow

color and was removed; this was followed later by entire excision of the embryo
from the endosperm, as described by Haynes (1959). The excised embryos were also
cultured on medium EC2, at 23°C and 16 h light. The rescued plantlets were
propagated and rooted in MS₃₀.

5

Results

Identification of amf-gene mutants
Based on iodine staining of microspores, genotypes corresponding tot nulliplex (no
10   wild-type GBSS-allele), simplex, duplex and triplex/quadruplex for the wild-type
GBSS allele were selected. This selection was according to the expected
segregation presented in Table 1.

Table 1. The expected and obtained offspring when duplex plants (AAaa x AAaa)
15   are crossed. These genotypes can be distinguished after iodine staining by their
segregation of blue and red microspores; triplex (AAAa) and quadruplex (AAAA)
plants where taken in one group. Genotypes with enough tubers to perform a field
trial were selected.

plant genotype	chance	microspore segregation blue:red	number of genotypes found[a]	number of gynotypes selected
aaaa	1/36	0:1	3	2
Aaaa	8/36	1:1	20	10
AAaa	18/36	5:1	33	11
AAAa	8/36	1:0	19	6
AAAA	1/36	1:0		

a: χ^2 (1:8:18:9) = 1.62 < 7.82 which indicates that the offspring is not deviating from
the expected 1:8:18:9 segregation of the gene-dosage genotypes for the wild-type
GBSS allele.

35

Starch granules of the duplex and triplex/quadruplex genotypes were completely
blue. In some of the simplex genotypes however, a small outer layer was red in a
small percentage of the starch granules. A number of tuberising plants belonging to
each gene-dosage group was selected for further research in a field trial (Table 1).

Table 2. The coagulated protein content analysis of offspring when duplex plants
(HZ91-RUG-025 x HZ91-RUG-075) are crossed. These genotypes were
distinguished after iodine staining by their segregation of blue and red
microspores; triplex (AAAa) and quadruplex (AAAA) plants where taken in one
group.

plant genotype	No. individuals	mean	S.E.
aaaa	15	1.51**	0.08
Aaaa	15	1.22	0.05
AAaa	17	1.10	0.12
AAAa	25	1.24	0.07
AAAA			

**indicates statistically significant effect P<0.05

Table 3. The coagulated protein content analysis of offspring when duplex plants (S90-1103 x S90-1101-0004) are crossed. These genotypes were distinguished after iodine staining by their segregation of blue and red microspores; triplex (AAAa) and quadruplex (AAAA) plants where taken in one group.

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plant genotype	No. individuals	mean	S.E.
aaaa	8	1.67**	0.08
Aaaa	13	1.38´	0.07
AAaa	17	1.25	0.12
AAAa	29	1.46	0.03
AAAA			

**indicates statistically significant effect P<0.05

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GBSS-protein content

The amount of GBSS-protein in the starch granule of different genotypes was analyzed. Fig. 1 clearly shows that the *amylose-free* plants had no GBSS in the starch granules, however no significant difference could be observed in the GBSS-

20   protein level of the other groups indicating that no dosage effect existed at the protein level. No differences in starch granule size and amylopectine and sucrose content of the tubers were found (data no shown)

Overexpression of heterologous protein in amf *mutant*

The invention furthermore provides a transgenic potato cell with at least one *amf-*

25   gene having been provided with a nucleic acid encoding a proteinaceous substance, a sink protein. In a preferred embodiment, said cell accumulates said sink protein up to more than 2%, preferably 4%, or even more than 5% to more than 7% of the total protein content of said cell. The protein preferably contains a high content of essential amino acids (preferably methionine, cysteine, lysine, threonine, or

30   tryptophane). To allow for an enhanced incorporation of these essential amino acids into a sink protein fraction of the *amf* potato cell said cell is provided with one or more gene constructs or nucleic acid molecules encoding at least one functional enzyme related to said amino acid's biosynthesis pathway allowing said cell to increasingly synthesise said amino acid, preferably wherein said amino acid is an

35   essential amino acid and thereby further regulates supply. Preferably, free amino

acid level is increased by introducing at least one gene encoding a feedback insensitive enzyme involved in biosynthesis of said amino acid. The over-produced free essential amino acids are trapped by incorporation in a sink protein, rich in said essential amino acid that is expressed at the same time in the plant.

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As food or feed organisms, or tissues, differ in limiting essential amino acids, the optimal amino acid content for a sink protein varies according to organism. A sink protein preferably is a protein specifically enriched in those amino acids for which a deficite occurs in the specific crop or organism. By producing the sink protein to at

10   least 2%, preferably to at least 4%, 5%, or even at least 7% of the total protein content of the tissue which is being used as food or feed, we compensate for the essential limiting amino acid. For example, for potato a sink protein preferably contains at least 5%, more preferably at least 10% lysine, at least 2.5% methionine, at least 2.5% cysteine, or at least 1.5% tryptophan.

15

The protein is stable in the plant, accumulates to high levels and has no drastic detrimental effects on the growth and physiology of the crop plant. The protein is well digestible by the livestock and/or human digestive tract.

20   Sink protein candidates can for example be selected from among known storage proteins. Several publications describe the amino acid composition of plant storage proteins, and their possible use to enhance the essential amino acids composition of food and feed crops. The storage proteins of cereal crops like wheat, barley and maize of the so-called prolamin type vary in their content of sulphur-containing

25   amino acids (methionine and cysteine). Some are relatively high in S-rich amino acids. However, most of them are severely deficient in lysine and tryptophan (Shewry, P. R., (1998) Transgenic Plant Research, p.135-149). The storage proteins in legumes and other dicotyledons are mainly of the globulin family or the albumin family. Globulins are generally very poor in the sulphur-containing amino acids,

30   but sometimes do contain a relatively high ratio of lysine. Vicilin (of Vicia faba) has a lysine content of 7.2%, threonine content of 3% and a methionine content of only 0.2%. The 2S albumin family of storage proteins in general have a high content of S-rich amino acids. Brazil nut 2S albumin contains ca. 26% sulfur amino acids (Ampe 1986), and sunflower 2S albumin (Sfa8) contains 24% sulfur amino acids

35   (Kortt 1991). Other storage proteins that have a high content of lysine residues are

the protease inhibitors C1 and C2 from barley (9.5% and 11.5% lysine respectively, Hejgaard and Boisen (1980)) and the cysteine protease inhibitor multicystatin of potato (Waldron et al., 1993 Plant Mol Biol. 23(4):801-12).

5   The level of protein accumulation in a plant is determined by the rate of synthesis in relation to the rate of degradation of this protein. The rate of degradation is determined by its sensitivity to attack of proteases that are present in the producing tissue. This protease sensitivity is influenced by the availability of susceptible sequence domains on the surface of the protein, in combination with the
10   structural rigidity of the protein. In order to select for a protein that will have a high chance to accumulate in the plant, the protein preferably has a rigid tertiary structure, with minimal exposed sequence domains. Certain proteins have a native tendency to aggregate into more or less regular or organised macromolecular structures, such as protein bodies or protein crystals. Naturally, storage proteins
15   that accumulate in plant tissue, where they have a storage function, are naturally adapted to remain stable in these plant tissues. Therefore seed storage proteins are distinct candidates to accumulate essential amino acids. However, few plant storage proteins will always have a desired composition relating to the desired essential amino acids. Furthermore, in general the amount of essential amino acids
20   is often too low. The invention herewith provides using sink protein that is encoded by a nucleic acid enriched with the necessary codons encoding said desired amino acids. In addition to this, in nature several proteins exist that form (semi)-crystalline structures in their natural tissue. Examples are some peroxisomal proteins like alcohol oxidase or urate oxidase, or crystallins (eye lens proteins). Also
25   plant structural proteins are able to form regular crystal like structures, for example the cysteine protease inhibitor multicystatin present in the peel of potato.

     As an example the use of a combination of gene constructs containing a DNA sequence encoding an enzyme having dihydrodipicolinate synthase (DHPS) activity
30   combined with a DNA sequence encoding a sink protein that is rich in essential amino acids, e.g. multicystatin, is provided. First half of this construct containing the DHPS genes results in an increased level of free lysine more than 10-fold the wild type level of each amino acid in a plant or parts thereof. The expression regulation should be such that expression occurs in such a way that lysine are
35   produced to a comparable extent without damaging the plant i.e. without causing

negative aberrations in the phenotype compared to wild type plants. The second
part of this combination of gene constructs consists of a gene encoding a sink
protein, which contains a high amount of essential amino acids. This sink protein
results in an increased incorporation of essential amino acids into the protein

5   fraction. As such it withdraws these amino acids from the pool of free amino acids,
thus further enhancing the synthesis of these essential amino acids.

Example 1. Chimeric gene construct with the mutant potato DHPS gene

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DNA isolation, subcloning, restriction analysis and DNA sequence analysis is
performed using standard methods (Sambrook, J. et al. (1989) Molecular Cloning. A
laboratory manual, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al.
(1994) Current protocols in molecular biology, John Wiley & Sons).

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In order to create a feedback insensitive DHPS, the evolutionary conserved amino
acid residue 134 (asparagine) were changed into a cysteine residue (WO0148230).
The mutant DHPS encoding DNA fragment (designated DHPS-134nc1) was used
for the expression in potato plants.

20   The chimeric gene containing the mutant DHPS gene was constructed by
subcloning DHPS cDNA from the pTriplex vector in pCR-Script SK(+) and from
this vector as a XbaI-Eco RI fragment in the pBluescript SK vector digested with
XbaI-EcoR. With this clone the mutagenesis was performed, resulting in clone
pAAP57-134nc1. At the 5'end the mutated DHPS cDNA was fused to a HindIII-SalI

25   fragment of the 800 bp long GBSS promoter fragment (Visser et al. ibid).
Downstream of the mutant DHPS sequence the termination signal of the nopaline
synthase gene from Agrobacterium tumefaciens was inserted (Greve, H.D. et al.
(1983) J.Mol.Appl.Genet. 1: 499-511) as an SstI-EcoRI fragment. The complete
chimeric gene was subcloned into the HindII-EcoRI sites of pBINPLUS (Van

30   Engelen, F.A. et al. (1995) Transgenic Research 4: 288-290) (pAAP105).

The binary vector pAAP105 was used for freeze-thaw transformation of
Agrobacterium tumefaciens strain AGLO (Höfgen, R. and Willmitzer, L. (1988)
Nucl.Acids Res. 16: 9877). Transformed AGLO was subsequently used for

35   inoculation of potato (Solanum tuberosum, variety Kardal) stem explants as

described by Visser (Visser, R.G.F. (1991) Plant Tissue Culture Manual B5 (ed. by
K. Lindsey): 1-9, Kluwer Acad. Publishers, The Netherlands). After shoot and root
regeneration on kanamycin-containing media plants were put in soil and
transferred to the greenhouse. Plants regenerated (on kanamycin-free media) from

5   stem explants treated with the Agrobacterium strain AGLO lacking a binary vector
served as a control.

Example 2 Overexpression of the potato multicystatin gene

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The Potato Multicystatin (hereinafter PMC) gene encodes a multidomain cysteine
protease inhibitor protein. A genomic clone of the PMC gene (Waldron et al., (1993)
Plant Molecular Biology, 23:801-812) was fused at the 5' end to the omega DNA
sequence from the coat protein of tobacco mosaic virus (Gallie, D.R. et al. (1987)

15   Nucl.Acids Res. 15: 3257-3273). Downstream of the PMC sequences the
termination signal of the octopine synthase gene from Agrobacterium tumefaciens
is inserted (Greve, H.D. et al. (1983) J.Mol.Appl.Genet. 1: 499-511). The chimeric
PMC gene construct is cloned as a BamHI/SpeI fragment in pBluescript. The
patatin promoter (Wenzler, H.C. et al. (1989) Plant Mol.Biol. 12: 41-50) is ligated as

20   a blunt (HindIII filled in)/BamHI fragment in front of the PMC chimeric gene
digested with SmaI/BamHI (pAAP169).

Example 3. Transformation of potato plants

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The binary vector pAAP105 and PAAP169 is used for freeze-thaw transformation of
Agrobacterium tumefaciens strain AGLO (Hofgen, R. and Willmitzer, L. (1988)
Nucl.Acids Res. 16: 9877). Transformed AGLO is subsequently used for inoculation
of tetraploid wildtype potato (Solanum tuberosum, variety Kardal) and tetraploid

30   amf mutant KA96-1396 stem explants as described by Visser (Visser, R.G.F. (1991)
Plant Tissue Culture Manual B5 (ed. by K. Lindsey): 1-9, Kluwer Acad. Publishers,
The Netherlands). After shoot and root regeneration on kanamycin-containing
media plants are put in soil and transferred to the greenhouse. Plants regenerated
(on kanamycin-free media) from stem explants treated with the Agrobacterium

35   strain AGLO lacking a binary vector serve as a control.

Example 4. Analysis of free amino acid content in transgenic plants

5   Tissue (0.5-1.0 gram) is homogenized with mortar and pestle in 2 ml 50 mM Pi-
     buffer (pH 7.0) containing 1 mM dithiothreitol. Nor-leucine is added as an internal
     standard. Free amino acids are partly purified by extraction with 5 ml of a
     water:chloroform:methanol mixture (3:5:12). Water phase is collected and the
     remaining re-extracted twice. After concentration by lyophilization to 3 ml, a 20 mu
10   l sample is analysed by HPLC using a cation-exchange column with post-column
     ninhydrine derivatisation of the amino acids detected at 570 and 440 nm
     (BIOCHROM 20, Amersham Pharmacia biotech).

Example 5. Coagulated protein content of transgenic plants

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     For determining raw and coagulated protein content, 300 grams of tuber material
     together with 1000 ppm sodium bisulphite was grinded in a laboratory blender,
     type Waring Blendor. To determine the dry matter content a homogeneous sample
     of approx. 10 gram was taken and dried overnight at 40°C. The rest of sample was
20   centrifuged for 10 min at 4600 rpm. Of the supernatant raw protein content was
     determined by determining nitrogen content with the Kjeldahl method and dry
     matter by overnight drying at 40°C. To determine the coagulated protein content in
     the supernatant the pH was adjusted to 5.2 with 19% HCl and the liquid was boiled
     for 1 minute. Subsequently, the samples were centrifuged for 10 min at 10000 rpm.
25   To remove the light substance the above liquid was filtered over an S&S 595 paper
     filter. Nitrogen content of the supernatant after the coagulation step was
     determined by the Kjeldahl method. All experiments were carried out in duplicate.

Table 4

5   LYSINE RICH

Vicilin	Fava bean	436aa	32 lys (7.2 %)	storage protein
SCR1	Soybean	102aa	21 lys (20.6%)	stress induced
Fcor 2	Strawberry	133aa	19 lys (14.3%	cold induced
TLRP	Tomato	62aa	11lys (17.7%)	matrix protein
multicystatine	Potato		11.8% lys	Protease inhibitor

METHIONINE RICH

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γZein	Maize	211 aa	55 met (26.1%)	storage protein
10kDa Zein		150aa	31 met (20.7%)	storage protein
2S albumin	Sunflower	141aa	18 met (12,8%)	storage protein

THREONINE RICH

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TIP13	Asparagus	182aa	23 thr (12.6%)	harvest
PTGRP	Tomato	78aa	16 thr (20.5%)	water stress

CYSTEINE RICH

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PA1b	Pea	130aa	10 cys (7.7%)	storage protein
SE60	Soybean	47aa	8 cys (17.2%)	storage protein
PCP1	Rape Seed	83aa	8 cys (9,6%)	pollen/stigma